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Full Name of Author — Nom complet de l'auteur

PAUL SMITH PASS

Date of Birth — Date de naissance	Country of Birth — Lieu de naissance
22/10/53	CANADA

Permanent Address — Résidence fixe

190 THE KINGSWAY,
TORONTO, ONTARIO,
M8X 2V6

Title of Thesis — Titre de la thèse

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1979	R.B. HODGETTS

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A Biochemical Analysis of Dopa Decarboxylase from Wild Type and Mutant Strains of *Drosophila melanogaster*

by

© Paul S. Pass

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA

FALL 1979

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled A Biochemical Analysis of Dopa Decarboxylase from Wild Type and Mutant Strains of Drosophila melanogaster submitted by Paul S. Pass in partial fulfilment of the requirements for the degree of Master of Science.

R.B. Hodgetts
Supervisor

J. Bell

L.D. Smillie

L.J. Hastings

Date... OCTOBER 19, 1979 ...

This thesis is dedicated to Annie and my parents

ABSTRACT

The enzyme dopa decarboxylase (DDC) from a wild type strain of *Drosophila melanogaster* was purified by a conventional method and characterized. The molecular weight of the native enzyme was determined to be 112,600 by sucrose gradient sedimentation and 102,200 by variable porosity polyacrylamide gel electrophoresis under non-denaturing conditions. The subunit molecular weight of the enzyme in the presence of SDS was 54,000. Based upon these data, the enzyme was postulated to be a homodimer.

The technique of immunoaffinity chromatography was used to purify cross-reacting material from the wild type and three mutant strains carrying putative DDC structural gene mutations. These strains were analyzed on polyacrylamide gels in an attempt to detect electrophoretic variation. Differences among the strains were not observed following electrophoresis under non-denaturing conditions or in the presence of SDS, but variation did appear to be evident in the presence of 8M urea. These preliminary experiments suggested that the mutant allele, *Ddc*^{5}, did indeed produce a subunit which demonstrated electrophoretic variation. This was taken as evidence that the mutation was in the structural gene for DDC.

Finally, a technique to radiolabel larval proteins *in vivo* was developed. This will circumvent a major difficulty encountered in this work, namely the insufficient quantity of purified DDC that could be obtained for biochemical

studies. The availability of radiolabelled enzyme should permit a variety of analyses on DDC in the mutant and wild type strains including the measurement of cross-reacting material, peptide mapping, and electrophoretic analysis.

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LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
BIS	N,N'-methylenebisacrylamide
BSA	Bovine serum albumin
CRM	Cross-reacting material
C.S.	Canton-Special
DDC	Dopa decarboxylase
DTT	Dithiotreitol
G-6-PD	Glucose-6-phosphate dehydrogenase
IA	Immunoaffinity
mRNA	Messenger RNA
MW	Molecular weight
PTU	Phenylthiourea
RMA	Rabbit muscle aldolase
SDS	Sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(Hydroxymethyl)amino methane
ul	Microlitre

I. INTRODUCTION

The regulation of gene expression is of fundamental importance to all living organisms. In particular, it has become apparent that the variable expression of the genetic potential over time comprises the basis for the development of higher organisms. The key to understanding the developmental process in higher eukaryotes is the elucidation of the mechanism by which a single totipotent cell develops into an organism composed of a variety of highly differentiated cells.

Our knowledge of genetic regulation in prokaryotes is based largely on the operon model of Jacob and Monod (1961). However, comparable progress has not yet been made towards understanding the regulation of gene expression during the development of higher eukaryotes.

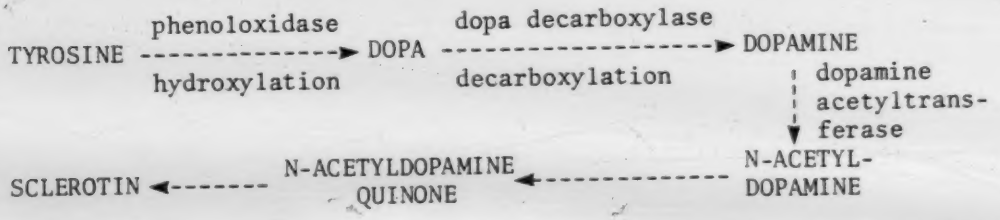
One approach in attempting to elucidate the mechanisms responsible for the development of higher eukaryotes has been to investigate, genetically and biochemically, systems which demonstrate variable expression during the course of development. One such system is the regulation of the enzyme dopa decarboxylase (DDC) throughout the development of the fruit fly, Drosophila melanogaster.

Interest in DDC arose initially from experiments on the blowfly, Calliphora erythrocephala, as the enzyme appeared to be under the direct influence of the steroid hormone, ecdysone (Sekeris and Karlson, 1964). Subsequent data (Shaaya and Sekeris, 1965) suggested that ecdysone was directly

involved with the induction of DDC only at puparium formation. Later in development, following pupation, ecdysone increased without a corresponding rise in enzyme activity. Still later, at adult eclosion, enzyme activity was high while ecdysone had returned to background levels. A more recent profile of ecdysone titre and DDC activity through the life history of Drosophila (Clark et al., 1979) has demonstrated five peaks of enzyme activity with only the major peak at puparium formation coinciding with a high titre of ecdysone. Thus, the regulation of this enzyme in Drosophila is complex and is being pursued in the hope that this system will provide insight into the regulation of gene expression in higher eukaryotes.

A. The Role of DDC in Insect Development

Although DDC has been shown to be involved in the production of neurogenic amines in the central nervous system of Drosophila (Dewhurst et al., 1972), the most widely studied function of this enzyme in insect development concerns its role in the sclerotization (hardening and darkening) of cuticles. The sequence of steps involved in the sclerotization pathway is diagrammed below.



Sclerotization in Calliphora was the subject of extensive

research by Karlson and his associates once the involvement of ecdysone in this process had been established. These workers determined that the sclerotization pathway is an alternate form of tyrosine metabolism which is activated, under the influence of ecdysone, in late third instar larvae. The end product of this pathway is N-acetyldopamine, the sclerotizing agent(Karlson and Sekeris,1962).

Several experiments indicated that the induction of DDC was the primary response to ecdysone. Firstly, the injection of ecdysone into the abdomens of larvae, isolated from the source of the hormone by ligature, was shown to enhance DDC activity substantially(Karlson and Sekeris,1962). Secondly, inhibitors of RNA and protein synthesis were found to inhibit the induction of DDC(Sekeris and Karlson,1964). This evidence prompted Sekeris and Karlson to postulate that the induction of DDC activity was the result of the de novo synthesis of the enzyme caused by the direct interaction of ecdysone with the DNA to stimulate the production of mRNA for the enzyme. More recent studies involving hormone receptor molecules(see O'Malley and Means,1974 and Yamamota and Alberts, 1976 for reviews) have shown that the mechanism postulated by Sekeris and Karlson is not correct, but their observation that ecdysone induces the production of both DDC and its mRNA in late third instar larvae has since been demonstrated in several insects(Chen and Hodgetts, 1974; Fragoulis and Sekeris, 1975a,b; Kraminsky, 1979).

Additionally, the stimulation of enzyme activity by

ecdysone has been established in the ovarian development of Aedes aegypti in which DDC was shown to be essential for the normal hardening and darkening of the chorion of mosquito eggs(Schlaeger and Fuchs,1974a,b). Dopa decarboxylase appears to be the only enzyme in the sclerotization process to be directly influenced by ecdysone as neither phenoloxidase nor dopamine acetyltransferase is dependent upon the presence of the hormone for activity(Sekeris and Karlson,1966).

The studies of DDC in Calliphora were followed by numerous reports on this enzyme in Drosophila. In 1969, Lunan and Mitchell found that DDC in Drosophila, as in Calliphora, is most abundant in the epidermal tissue. These authors reported that the sclerotization pathway in the two insects is virtually the same with the exception of the accumulation of tyrosine-o-phosphate in late larval stages of Drosophila. They suggested that this compound is an inert storage product that is rapidly converted to tyrosine when sclerotization is initiated.

E. Genetical and Biochemical Studies on Dipteran DDC

The advantage of studying DDC in Drosophila melanogaster becomes apparent as a result of the genetic analysis which has been carried out by two groups, those of Wright and Hodgetts.

Wright initially approached the genetic study of DDC by using alpha-methyl dopa, a non-competitive inhibitor of the

enzyme(Sparrow and Wright, 1974) that is a structural analogue of dopa, to screen for mutants affecting the level of DDC activity. He rationalized that one might expect to recover mutants with altered levels of DDC which would make them resistant or more sensitive to the inhibitor than the wild type.

A search for alpha-methyl dopa resistant strains(Sherald and Wright, 1974) ultimately yielded three variants, designated S, R, and R2, which were analyzed in detail. Two of the strains, S and R, showed substantial increases in DDC activity, relative to the wild type, accompanied by increased resistance to the inhibitor. The genes responsible for these variations were localized using compound second chromosomes in which portions of the wild type chromosome were combined with portions of the chromosome derived from the mutants. The region responsible for increased DDC activity was localized to chromosome 2L while the alpha-methyl dopa resistance mapped to both 2L and 2R. The third strain, R2, showed increased resistance to alpha-methyl dopa, again mapping to 2L and 2R, without any variation in DDC activity relative to the wild type.

A screen for alpha-methyl dopa hypersensitive mutants(Sparrow and Wright, 1974) recovered eleven, but no variation could be detected with respect to DDC activity, thermolability, or <u>in vitro</u> inhibition by alpha-methyl dopa. Thus, attempts to use alpha-methyl dopa to screen for DDC activity variants were only partly successful.

Hodgetts(1975) used the technique of segmental aneuploidy(Lindsley et al.,1972) to identify a single dosage sensitive region for DDC on chromosome 2L between bands 36EF and 37D. This permitted the subsequent recovery and analysis of a set of overlapping deficiencies for the DDC dosage sensitive region(Wright et al.,1976a). Ethyl methanesulfonate-induced recessive lethal mutations in this region were isclated(Wright et al.,1976b) using one of the deficiencies known to include the dosage sensitive region. Eight of these mutants in the heterozygous state exhibited DDC activity only 55% that of the wild type. These were designated $l(2)Ddc^{1}$ to $l(2)Ddc^{8}$. Wright(1977) concluded that the substantially lower DDC activity demonstrated by these mutants in conjunction with the observation that the enzyme produced is more thermolabile than the wild type enzyme provided strong evidence that these were DDC structural gene mutants. Since these putative DDC structural gene mutants complemented all eleven alpha-methyl dopa hypersensitive mutants ($l(2)amd^{H}$) and did not show hypersensitivity to alpha-methyl dopa, Wright concluded that two distinct, virtually adjacent, genes existed producing gene products which were possibly related in function.

The studies of Fragoulis and Sekeris(1975c), which indicated that DDC purified from Calliphora larvae was a heterodimer with subunit molecular weights of 46,000 and 50,000, led Wright et al.(1976b) to suggest that the products of the $l(2)Ddc^{+}$ and $l(2)amd^{+}$ loci might function as

catalytic and regulatory subunits, respectively, of the enzyme.

C. Purpose of this Research

When this study was initiated, the biochemical information concerning DDC in Drosophila melanogaster was minimal. However, the genetic studies had led to hypotheses which demanded biochemical analyses of the enzyme.

A major goal of this research was to investigate the subunit structure of DDC from wild type Drosophila. As stated above, Fragoulis and Sekeris (1975c) had claimed that DDC from Calliphora was a heterodimer. A preliminary result of Hodgetts and Clark which suggested that DDC from Drosophila was also a heterodimer was reported by Wright et al. (1976b). However, the molecular weight analysis in this study (which has been reported by Clark et al., 1978) showed that the enzyme in Drosophila is a homodimer.

Based upon the finding that DDC from Drosophila is a homodimer, Clark et al. (1978) proposed that the DDC activity and cross-reacting material (CRM) levels which they observed in the l(2)Ddc mutants isolated by Wright et al. (1976b) could be accounted for if these were mutations in the structural gene for the enzyme. Specifically, they divided the putative structural gene mutants into three classes based upon DDC activity and CRM levels. Figure 1 illustrates the molecular nature of each class.

Class I is composed of true null mutants. Chromosomes

Figure 1. A hypothesis to explain the DDC-deficient mutants, $\underline{1(2)Ddc}^{1}$ through $\underline{1(2)Ddc}^{8}$, in terms of mutations in the structural gene for DDC(Clark et al., 1978)

CLASS	GENOMIC REPRESENTATION	SUBUNITS PRODUCED		POSSIBLE DDC DIMERIC MOLECULES	CRM (total)	ENZYME ACTIVITY (total)
I	$\frac{Ddc^{2}/Ddc^{3}/Ddc^{7}}{Ddc^{+}}$ I / CyO	The mutant I chromosome produces incomplete polypeptides	+	+ / +	normal (50%)	normal (50%)
II	$\frac{Ddc^{1}/Ddc^{4}/Ddc^{6}}{Ddc^{+}}$ II / CyO	mutant II		1 mutant II / mutant II	none	none
			mutant II	2 mutant II / +	slight	slight
		+	+	1 + / +	normal (37-42%)	normal (28%)
III	$\frac{Ddc^{5}/Ddc^{8}}{Ddc^{+}}$ III / CyO	mutant III		1 mutant III / mutant III	none	none
			mutant III	2 mutant III / +	normal	slight
		+	+	1 + / +	normal (75-79%)	normal (31-36%)

carrying a class I allele give rise to subunits which are incapable of dimerization and are probably degraded. Such alleles behave like deletions or nonsense mutations in the structural gene for the enzyme. Consequently, in the heterozygous mutant stocks both the enzyme activity and CRM levels are 50% of the wild type as only the CyO chromosome(CyO is a multiply inverted balancer chromosome which carries the 1(2)Ddc$^+$ allele) will ultimately produce DDC wild type subunits. A class II mutant results in altered enzyme subunits being produced from the chromosome possessing the mutant allele. The class II subunits can associate with wild type subunits produced by the CyO chromosome. The phenomenon of negative complementation is alleged to occur in this situation in which a mutant II/wild type enzyme molecule has reduced activity and CRM relative to a wild type/wild type molecule. Therefore, less than 50% activity and CRM are observed. Finally, the class III mutants are analogous to those in class II except that their mutations are not as severe. This is evident from the substantially higher CRM levels, 75% of the wild type, implying that the mutant III/wild type enzyme molecule has virtually wild type CRM levels in addition to some enzyme activity.

A second goal of this thesis was to provide additional biochemical evidence for classifying the 1(2)Ddc mutants into the three groups just described. The demonstration that a putative structural gene mutant produces a protein that is

biochemically distinguishable from the wild type protein is considered to be strong evidence that, in fact, the mutation does occur in the structural gene. With this end in mind, the development of a purification procedure involving immunoaffinity chromatography(IA) became essential. Such a procedure allowed for the isolation of CRM-positive, but not necessarily active, DDC. Based upon the observations of Clark et al. (1978) that the class III mutants possess 75% CRM, the IA technique was used to purify both wild type and mutant subunits of the enzyme from these stocks. The purified enzyme was then subjected to electrophoresis on analytical polyacrylamide gels in an attempt to detect electrophoretic variation between enzyme derived from the wild type and enzyme derived from the class III putative structural gene mutants.

II. MATERIALS AND METHODS

A. Maintenance of Stocks

The strains analyzed in these experiments included Canton-Special (C.S.), the wild type, and two strains in which the DDC mutant alleles 1(2)Ddc^{5} and 1(2)Ddc^{8} were present. More specifically, the strains were b hk 1(2)Ddc^{ns} pr/CyO, dp^{lv1} Cy pr cn^{2}, designated Ddc^{5}/CyO in this thesis, and rdo hk 1(2)Ddc ^{ns}/CyO, dp ^{lv1} Cy pr cn^{2}, designated Ddc^{8}/CyO. The doubly heterozygous strain, Ddc^{5}/Ddc^{8}, was produced by crossing Ddc^{5}/CyO x Ddc^{8}/CyO since these two mutant alleles complement one another (Wright et al., 1976b).

Large quantities of late third instar larvae were collected from population cages containing several thousand adult flies on 21x14x7cm plastic trays containing standard food (Nash and Bell, 1968). Egg laying was not critically timed although trays were usually left within the cage for about one day at 25°C. Spreading a live yeast and sucrose paste on the surface of the food was found to increase egg laying greatly. Once removed from the population cage, the organisms were allowed to develop within the trays at 25°C until late third instar. The mature larvae were subsequently collected from the walls of the trays, dried, weighed and frozen at -40°C.

B. Enzyme Assay

The radiometric method for assaying DDC activity was performed in the manner fully described by Clark et al. (1978). Standard conditions, including that of a two hour post incubation period, were followed.

C. Protein Determination

The protein concentration in all instances was determined by the method of Lowry et al. (1951). Bovine serum albumin was used to generate a new standard curve for each determination. Sodium tartrate was found to produce a more linear standard curve than sodium citrate and was therefore used throughout these experiments.

D. Enzyme Purification

Dopa decarboxylase was purified by one of two methods. Initially, the purification procedure followed was that outlined by Clark et al. (1978). However, this procedure was ultimately replaced by one which included immunoaffinity chromatography. The development of the immunoaffinity chromatography step will be discussed at length in the Results.

E. Concentration of Highly Purified Enzyme

The concentration of highly purified DDC was accomplished at the outset of this work using an Amicon Model 10-PA propellent-pressurized, non-agitated

ultrafiltration cell equipped with a 25mm XM-50 membrane. However, the later use of stirred ultrafiltration devices provided far more satisfactory results. Enzyme from the strains used for mutant analysis was initially concentrated in an Amicon Model 52 stirred ultrafiltration cell using a 43mm YM-10 membrane. The Model 52 concentrate was then transferred to an Amicon Model 3 stirred cell with a 25mm UM-10 membrane in which the concentration procedure was completed. Final concentrates reached volumes as low as 200ul.

F. Sucrose Gradient Sedimentation

Molecular weight estimates for DDC were obtained using the method of Martin and Ames(1961), by sedimentation through a 5 to 20% linear gradient of sucrose in 0.032M sodium phosphate buffer(pH 7.3) containing 1.2×10^{-4}M pyridoxal-5'-phosphate and 10^{-3}M DTT. Centrifugation was at 150,000xg for 18hr at 5°C in a Beckman SW 50.1 rotor. Gradients were unloaded by collecting drops into tubes containing 200ul of the above buffer. The protein standards used in these runs included bovine serum albumin, monitored by its absorbancy at 280nm, and alcohol dehydrogenases from horse liver and yeast, assays for which were carried out as described by Vallee and Hoch(1955) with minor modifications.

G. Polyacrylamide Gel Electrophoresis

Non-denaturing Gel Electrophoresis

Analytical polyacrylamide gels were prepared according to Hedrick and Smith(1968) with the addition of 0.062%(v/v) TEMED(Eastman Chemical Co.) to the large pore gel solution to effect polymerization.

The molecular weight studies for the non-denatured, wild type enzyme were carried out using 8.5% and 10.5% polyacrylamide gels cast in tubes. The gels were run at 1.5ma/tube for 3 to 3.5hr at 4°C. Data from these runs were analyzed as suggested by Hedrick and Smith(1968).

Non-denaturing 10% polyacrylamide gels for the analysis of the mutants were prepared as slabs(14x11x0.1cm) and run at 12ma for 5hr at 4°C. The reservoir buffer was replaced with fresh buffer at 1.5hr intervals throughout each run.

Denaturing SDS Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gels were run as described by Kikuchi and King(1975) to study the subunit size of DDC for the wild type and mutant strains.

The molecular weight estimates of subunits of the wild type enzyme were based upon runs in slab gels(12ma) or in tubes(1.5ma/tube) for 5hr at room temperature. Samples were diluted 1:1 with reducing buffer(3% SDS, 5% 2-mercaptoethanol, 10% glycerol, 62.5mM Tris-HCl, pH 6.8) and boiled for 5min prior to loading. The data were analyzed according to Weber and Osborn(1969).

Denaturing Urea Gel Electrophoresis

Polyacrylamide urea gels were prepared according to the protocol of Panyim and Chalkley(1969) with some modifications. A 10% acrylamide gel solution at pH 4.1 containing 8M urea(pre-purified by Dowex anionic and cationic exchange resins), 5% glacial acetic acid, a BIS:acrylamide ratio of 1:150 and 0.33%(v/v) TEMED polymerized with the addition of 0.5%(w/v) ammonium persulfate. The gels were run at 150V for 12hr at 4°C. Loading samples in 8M urea containing 5% glacial acetic acid proved to be superior to the preparation suggested by Panyim and Chalkley(15% sucrose containing 5% glacial acetic acid).

Protein Visualization in Gels

All gels were stained overnight in Coomassie Brilliant Blue R(0.05% in 25% isopropyl alcohol, 10% acetic acid). Slab gels were destained by diffusion in 7% acetic acid for at least one day. Tube gels were destained electrophoretically and, in some cases, scanned with a Joyce microdensitometer. Complete instructions for preparing all gels are located in the Appendix.

H. Amino Acid Analysis

Dopa decarboxylase of 98% purity was dialyzed against double distilled water, lyophilized and subsequently hydrolyzed in 6N HCl at 110°C *in vacuo* for periods of 24, 48 and 72hr. Amino acid analysis of this material was performed using the Durrum Model D-500 amino acid analyzer. These

analyses were kindly carried out by M. Nattriss.

I. Preparation of the Immunoaffinity Matrix for Chromatography

Partial Purification of Antibody

The IgG component was purified from the crude antiserum prepared by Clark et al. (1978) by a method adapted from Andres (1976). A variable volume (typically 2 to 5 ml) of crude antiserum (batch 6GD6) was diluted 1:1 with phosphate buffered saline (0.7% NaCl in 0.01M potassium phosphate, pH 7.5). Subsequently, 0.5g/ml of solid ammonium sulfate was added and the precipitated protein collected by centrifugation at 12,000xg for 10min at 4°C. The pellet was resuspended in a volume of phosphate buffered saline one half that of the previous volume added, and desalted by passage over a 1.2x45cm Sephadex G-25 column which had been equilibrated with 0.01M potassium phosphate, pH 7.5. Fractions in which the optical density at 280nm was greater than 0.2 were pooled and applied to a 1.2x10cm DE32 (Whatman Biochemicals Ltd.) column equilibrated with 0.01M potassium phosphate, pH 7.5. All protein eluting in the initial 25 ml of eluate was concentrated to 5 ml by ultrafiltration using the Amicon Model 52 stirred cell equipped with a 43mm PM-10 membrane.

The concentrated, partially purified antibody was titred in the following manner. A crude extract of late third instar C.S. larvae was prepared at a concentration of

100mg/ml in 0.05M Tris-HCl buffer (pH 7.3) which included 1mM PTU. A 100ul volume of crude extract was mixed in equal proportion with an appropriate dilution of the partially purified antibody. The mixture was then incubated in an ice bath for 2hr. Following the incubation period, all samples were centrifuged at 27,000xg for 10min and the supernatant was assayed for enzyme activity. A sample of the initial larval crude extract was also assayed as a control following 2hr on ice. Effective immunoaffinity columns were prepared from IgG in which an equivalence point dilution of 1:50 or greater was observed.

Activation of Sepharose 4B by Cyanogen Bromide

The activation of Sepharose 4B(Pharmacia Fine Chemicals) by cyanogen bromide(Eastman Kodak Co.) was accomplished by the method of March et al.(1974). Sepharose 4B is prepared for commercial use as an aqueous suspension in 0.02% sodium azide. It was washed free of sodium azide by mixing 20ml of the commercial preparation with a large volume of double distilled water, allowing the agarose beads to settle and then decanting the wash water. Subsequently, 20ml of double distilled water was added to the washed Sepharose followed by the addition of 40ml of 2M sodium carbonate, while mixing slowly at room temperature. The stirring was increased just prior to the addition of 4ml of an acetonitrile solution of cyanogen bromide(2g of cyanogen bromide/ml acetonitrile). Vigorous stirring of this mixture proceeded for 1 to 2min after which the slurry was poured

onto a 9in diameter Buchner funnel equipped with Whatman #1 filter paper underlaid with 50u Nitex nylon mesh. Under gentle suction, the activated agarose beads were thoroughly washed with 500 to 1000ml each of 0.1M sodium bicarbonate(pH 9.5), double distilled water, and 0.2M sodium bicarbonate(pH 9.5), respectively. The slurry was allowed to filter under vacuum until a compact cake had formed at which time it could be coupled to the IgG.

Coupling of Partially Purified Antibody to Activated Sepharose 4B

The activated Sepharose described above was transferred to a 250ml plastic bottle containing 20ml of 0.2M sodium bicarbonate(pH 9.5) and the 5ml of concentrated, partially purified antibody. The coupling reaction was permitted to occur for 20hr at 4°C with gentle agitation. Following this, a protocol adapted from Edwards et al. (1977) was followed to the conclusion of the matrix preparation. Specifically, 45ml of 1M glycine in 0.2M sodium bicarbonate(pH 9.5) was added with the intention of blocking any unreacted sites on the agarose beads which would otherwise continue to have coupling potential.

Finally, the antibody-Sepharose matrix was washed in the following sequence of steps, with each of the first three wash buffers containing 0.5M NaCl: 200ml of 0.1M sodium bicarbonate(pH 9.5), 400ml of 0.1M sodium acetate(pH 4.0), 200ml of 0.1M sodium bicarbonate(pH 9.5) and 400ml of Buffer P(0.01M sodium phosphate buffer(pH 7.3) containing

1.3mM 2-mercaptoethanol, 1mM EDTA and 0.01% sodium merthiolate). Buffer P is intended for storage purposes and the immunoaffinity matrix was maintained in this buffer at 4°C for at least one year without appreciable loss of activity.

J. Peptide Mapping

Preliminary two-dimensional peptide mapping experiments were performed according to the protocol of Bates *et al.*(1975) using rabbit muscle aldolase(RMA). Prior to tryptic and chymotryptic digestion, RMA was S-carboxymethylated as described by Gibbons and Perham(1970). The digest was analyzed by electrophoresing at 500V for 20min at 4°C(pyridine- acetic acid-water 25:1:225) in one dimension followed by thin layer chromatography(butan-1-ol-acetic acid -water-pyridine 15:3:12:10) in the second dimension. The resulting peptide map was visualized by dipping the cellulose paper in a ninhydrin-cadmium solution(see the Appendix for details) followed by oven drying.

K. *In Vivo* Labelling of Larval Proteins

Radioactive labelling of larval proteins was performed by feeding 50mg of third instar larvae(28 to 40 larvae/50mg, depending upon age) 75ul of an aqueous solution containing a ^{14}C amino acid mixture (Amersham code CFB.104, batch 54) in covered glass petri dishes(3cm diameter x 1cm height). The

commercially available preparation had a radioactive concentration of 50uCi/ml prepared in 2% ethanol and was used directly or diluted with double distilled water, depending upon the amount of incorporation desired. The larvae had usually ingested all of the solution within 5hr, although this time varied with larval age. Once all of the radioactive mixture had been consumed, the addition of 100ul of 1% sucrose kept the larvae active until extraction at 10hr. Crude extracts were prepared at 4°C consisting of 50mg of larvae/ ml of extraction buffer(0.05M Tris-HCl, pH 7.3 at 22°C, containing 1mM PTU).

The time course experiment shown in the graph in Figure 11a of the Results required that the crude extracts be TCA-precipitated on Whatman 3MM filter papers, according to the prctocol of Mans and Novelli (1961), and counted by liquid scintillation. The photograph in Figure 11b shows a series of labelled crude extracts which had been electrophoresed in the presence of SDS and fluorographed as described by Bonner and Laskey(1974).

III. RESULTS

A. Physical Properties of the Wild Type Enzyme

Dopa decarboxylase from mature larvae of the Canton-S. strain was purified for the early analytical work using the technique described by Clark et al. (1978). This approach will be referred to as the conventional method for purifying the enzyme.

Molecular Weight of the Native Enzyme

Molecular weight estimates were obtained by sucrose gradient sedimentation and variable porosity non-denaturing polyacrylamide gel electrophoresis on DDC purified by the conventional method.

The mean molecular weight of DDC based upon sucrose gradient sedimentation experiments was 112,600±6,200 (Table 1). No difference in the sedimentation behaviour of the enzyme was observed in the presence of 0.3M NaCl in contrast to a previous report on DDC from Calliphora (Fragoulis and Sekeris, 1975c). These authors observed several species of varying molecular weights in the absence of salt, but only one species in the presence of 0.3M NaCl. They attributed this result to an aggregation-dissociation effect which was dependent upon a low salt condition.

Experiments using variable porosity non-denaturing polyacrylamide gel electrophoresis indicated a mean molecular weight of 102,200±9,100 for DDC (Figure 2). This estimate corresponds well with that obtained from sucrose

Table 1. Sucrose gradient sedimentation analysis of dopa decarboxylase

PROTEIN STANDARD		MOLECULAR WEIGHT	$S_{20,w}$
Bovine serum albumin[1]		67,000	4.4
Horse liver ADH[2]		80,000	4.8
Yeast ADH[3]		150,000	6.7
DOPA DECARBOXYLASE			
	Experiment 1		
Gradient			
1	Bovine serum albumin + DDC	107,765	6.05
2	Horse liver ADH + DDC	108,641	5.89
3	Yeast ADH + DDC	114,225	5.60
	Experiment 2		
Gradient			
1	Bovine serum albumin + DDC	108,199	6.07
2	Horse liver ADH + DDC	112,742	6.03
3	Yeast ADH + DDC	124,047	5.92
	Average	112,600 ± 6,200	5.93 ± 0.18

Molecular weights of dopa decarboxylase were determined by comparing the sedimentation behaviour of the enzyme to that of a standard protein included in the same tube, using the relationship: $MW_1 = (d_1/d_2)^{3/2}MW_2$, where d_1 and d_2 are the distances sedimented of the unknown and standard, respectively (Martin and Ames, 1961).

Sedimentation co-efficients were calculated according to the equation: $S_1 = (d_1/d_2)S_2$ (Martin and Ames, 1961). The molecular weights and sedimentation co-efficients of the standards were taken from the following references:

1 Loeb and Scheraga, 1956.
2 Drum et al.,1967.
3 Hayes and Velick, 1954.

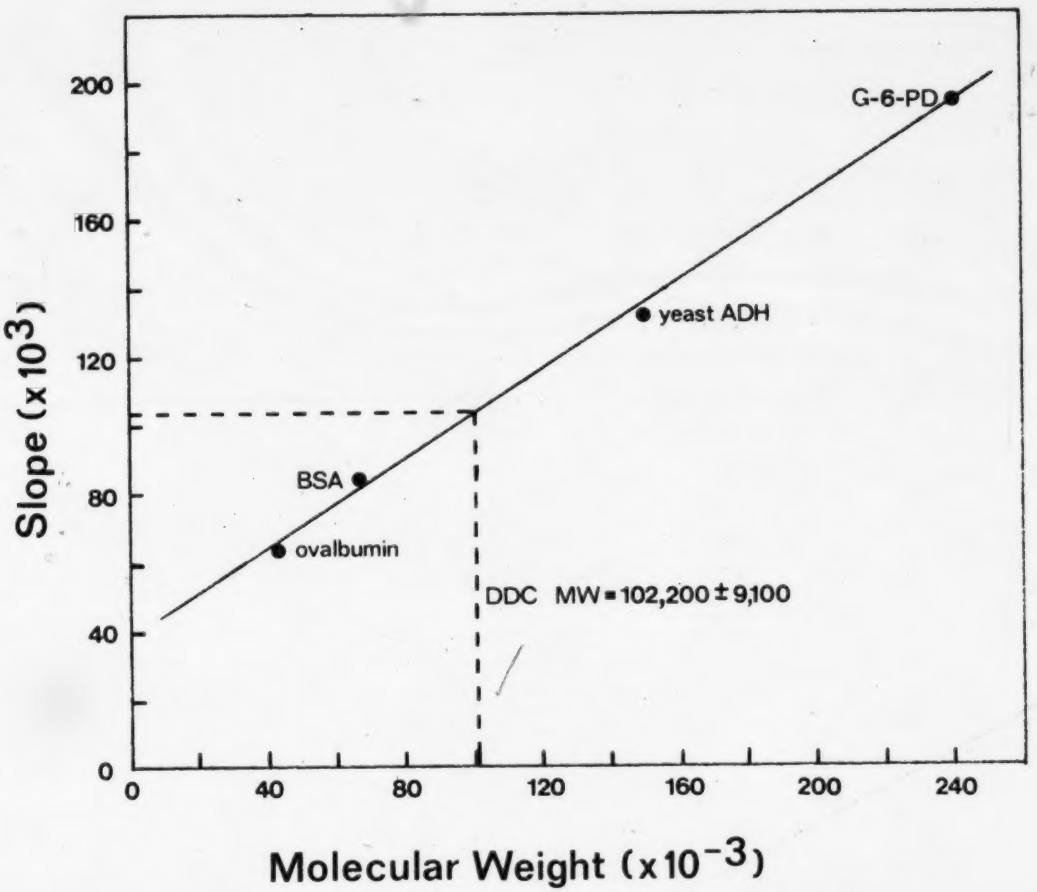


Figure 2. Molecular weight of DDC using variable porosity non-denaturing polyacrylamide gel electrophoresis

Polyacrylamide gels of 8.5% and 10.5% cast in tubes were prepared as described in the Materials and Methods. The data were plotted according to Hedrick and Smith(1968). Three separate sets of runs were made and the standard curve shown in the figure was fitted to the averaged data points by the method of least squares. Five estimates of the molecular weight of DDC were obtained in the three experiments and the mean value with its standard deviation is shown in the figure. The molecular weights of the standards were taken from the following sources: G-6-PD, Yoshida(1966); yeast ADH, Hayes and Velick(1954); BSA, Loeb and Scheraga(1956); ovalbumin, Edsall(1953).

gradient sedimentation.

Several attempts were made to determine the molecular weight of the native enzyme in the Beckman Model E ultracentrifuge. However, the small quantity of DDC present in *Drosophila* and the losses associated with concentrating dilute enzyme solutions prevented the attainment of sufficient DDC for analysis. Improvements in the purification procedure and ultrafiltration have since made a Model E estimate more feasible.

Subunit Molecular Weight

Dopa decarboxylase migrated as a single molecular species in SDS polyacrylamide gels, exhibiting a molecular weight of 53,950 when run on a slab gel(Figure 3). The subunit molecular weight was also estimated from gels cast in tubes, under the same conditions as described in Figure 3, and found to be 54,450.

Amino Acid Analysis

The partial amino acid composition of the enzyme is presented in Table 2. This analysis was of particular benefit in the preparation of *in vivo* labelled DDC, which was accomplished by feeding larvae radiolabelled amino acids. The mixture of amino acids to be used in such a preparation may thus be primarily composed of the amino acids most common to DDC as determined by the amino acid analysis.

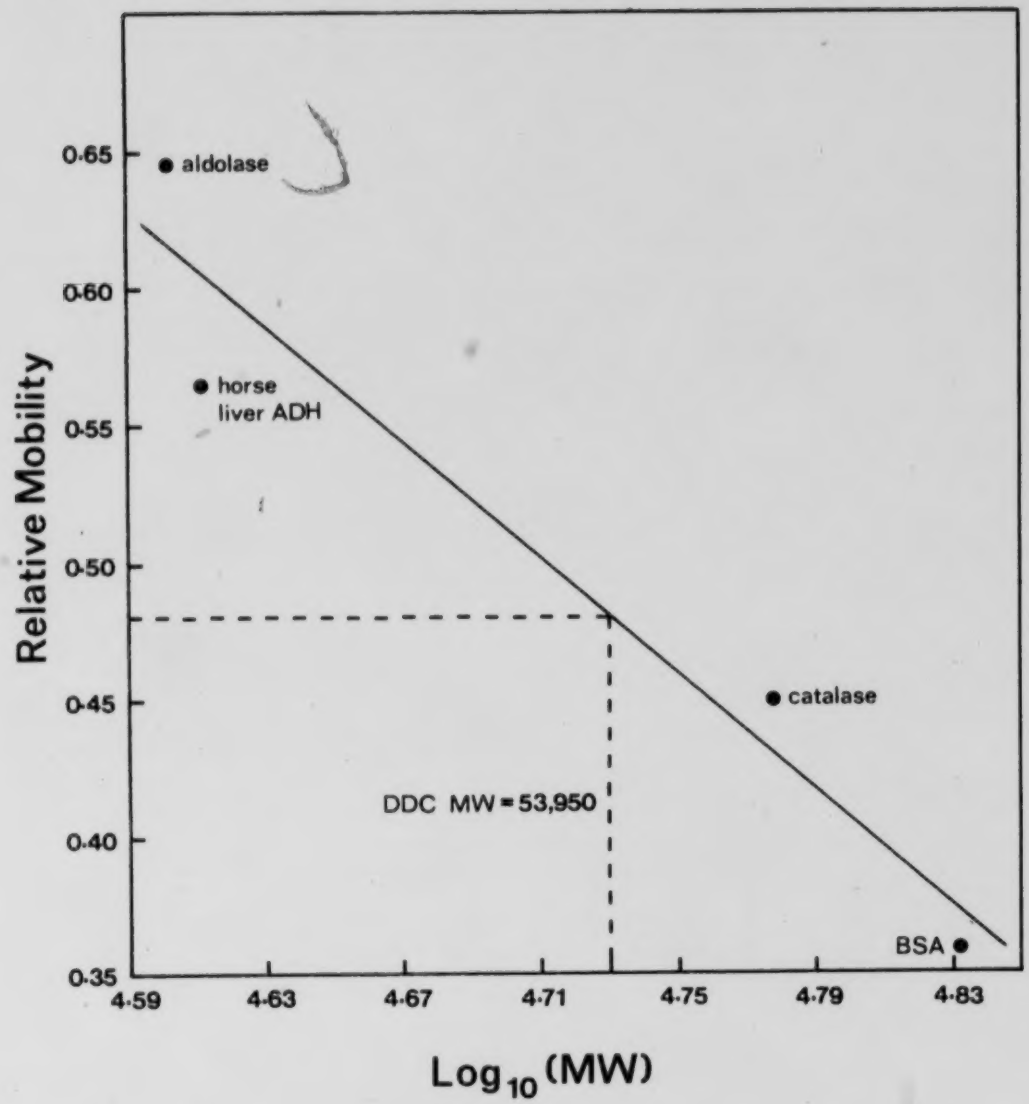


Figure 3. Subunit molecular weight of DDC

A 10% denaturing SDS polyacrylamide gel was run as described in the Materials and Methods and the data analyzed according to Weber and Osborn(1969). Each relative mobility value is the average of duplicate samples. The molecular weights of the subunits of the standard proteins were taken from Weber and Osborn(1969), and the standard curve was fitted by the method of least squares.

Table 2. Amino acid composition of dopa decarboxylase

AMINO ACID	RESIDUES[a]/SUBUNIT
Glycine	73.9
Glutamic acid[b]	60.6
Serine[c]	46.9
Aspartic acid[b]	40.3
Alanine	40.0
Threonine[c]	38.5
Leucine	35.8
Lysine	34.8
Valine[d]	23.6
Histidine	22.2
Proline	19.5
Arginine	18.4
Isoleucine[d]	17.9
Phenylalanine	16.0
Tyrosine	13.8
Methionine	9.0

Details of the hydrolysis are given under Materials and Methods. At least two separate samples were hydrolyzed at 24, 48 and 72 hr. Except where noted, each value is the average of data obtained at the three hydrolysis times. Cysteine and tryptophan were not determined.

[a]Calculated on the basis of subunit molecular weight of 54,000.

[c]Estimated by extrapolation to zero time of hydrolysis.

[d]Calculated from 72 hr hydrolysis.

[b]Includes both acid and amide forms.

B. Purification of DDC by Immunoaffinity Chromatography

Following the characterization of the wild type enzyme, a study was undertaken to determine the nature of the putative DDC structural gene mutants discovered by Wright et al. (1976b). However, to perform such an analysis, a technique was required to purify mutant enzyme which was CRM-positive, but not necessarily active. The recovery of highly purified DDC by the conventional purification procedure provided the basis for the preparation of an immunoaffinity matrix that allowed a purification of CRM-positive enzyme. This procedure is described at length in the text which follows.

Operation of the Immunoaffinity Column

The details of running the IA column are provided in the legend accompanying Figure 6 (see below). An explanation of the various steps follows.

A layer of 6.2ml of the antibody-Sepharose matrix, prepared as described in the Materials and Methods, was placed on top of 77ml of Sephadex G-25 in a column with a diameter of 1.2cm. Once the column had been equilibrated, an alumina eluate from the conventional procedure was applied. A buffer containing 1M NaCl was then passed over the column to elute proteins non-specifically bound to the antibody-Sepharose. This relatively low salt buffer was incapable of eluting DDC specifically bound to the antibody. Subsequently, a buffer containing 10% glycerol washed away any remaining NaCl and non- specifically bound proteins and

provided a stable collecting buffer for the enzyme once it had been eluted. A note of potential convenience in running the column is the observation that no significant loss of enzyme activity occurred when antibody-bound enzyme remained on the column bathed in the 10% glycerol buffer for up to one day. Thus, a column run could be started one day and completed the next provided that the run was stopped during the application of the 10% glycerol buffer. Elution of the enzyme was accomplished with a buffer containing 3M NH_4SCN which dissociated the enzyme from the antibody. The importance of having a long column of Sephadex G-25 immediately below the antibody-Sepharose column must be stressed. The eluted enzyme, which is sensitive to high salt, must be quickly separated from the 3M NH_4SCN by the G-25. A 3% solution of $FeCl_3$ was used to demonstrate the initial appearance of the SCN^- ion in fraction 77(M.T. Yang, personal communication). Since the pure enzyme was present in fractions 67 to 74, the column of G-25 did successfully separate DDC from the high salt.

Immediately following the elution of the pure enzyme, 50ul samples were assayed from each relevant fraction. The fractions containing enzyme activity in the vicinity of fractions 67 to 74 were then pooled and glycerol was added to approximately 10% by volume to ensure stability of the enzyme during the subsequent freezing at -40°C. The eluate could be thawed at a later time and concentrated to smaller volumes by ultrafiltration as described in the Materials and

Methods. Dopa decarboxylase purified by the immunoaffinity technique is shown in Figure 4 in comparison with DDC purified by the conventional method.

Maximization of Column Efficiency

The material routinely loaded on the IA column was a crude larval extract purified through the alumina gel step of the conventional purification procedure. The extent of purification usually obtained by this approach can be seen by comparing slots 1 and 3 in Figure 5. Slot 1 shows the spectrum of proteins in an alumina eluate and slot 3 the purified enzyme eluted from the column. The necessity of applying a partially purified preparation to the IA column is evident by comparing slots 3 and 4. Slot 4 shows that the direct application of a crude extract to the IA column resulted in several protein species contaminating the fractions where pure DDC is recovered.

Once the requirement for a preliminary purification to the alumina step had been established, the efficiency of the column was maximized for the recovery of DDC activity. The maximum capacity of the column was ascertained by loading it with decreasing amounts of alumina eluate until enzyme activity was no longer evident in the effluent during the wash procedure. The elution profiles shown in Figure 6(see below) indicate that unbound enzyme typically appeared in the wash at about fraction 30 and slot 2 of Figure 5 shows that several other unbound protein species eluted in this region. The bound enzyme was eluted in fraction 68.

7

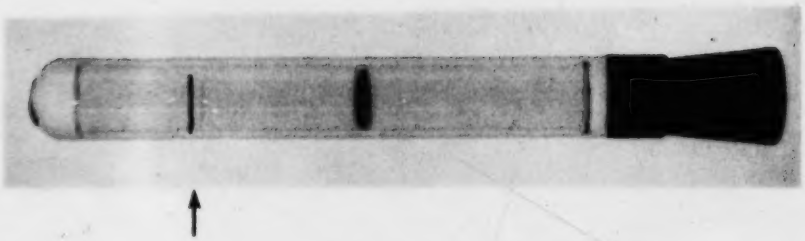
Figure 4. Electrophoresis of DDC purified by the conventional and immunoaffinity methods

a. Approximately 5 µg of enzyme purified by the conventional method were loaded onto an 8.5% non-denaturing gel and electrophoresed at 1.5 ma/tube for 3.5 hr at 4°C. The purity of this preparation was estimated to be at least 98% by microdensitometer scanning. The arrow denotes the location of the tracking dye.

b. Approximately 5 µg of enzyme from the eluate of the immunoaffinity column were loaded onto an 8.5% non-denaturing gel and electrophoresed at 2 ma/tube for 3 hr at 4°C. A second tube loaded with the same material was not stained, but sliced, and each slice assayed for DDC activity. A single peak of enzyme activity was detected whose position corresponded with the single band evident in the photograph. Microdensitometer scanning indicated that the purity of this preparation was also at least 98%. The arrow again denotes the location of the tracking dye.

a

b



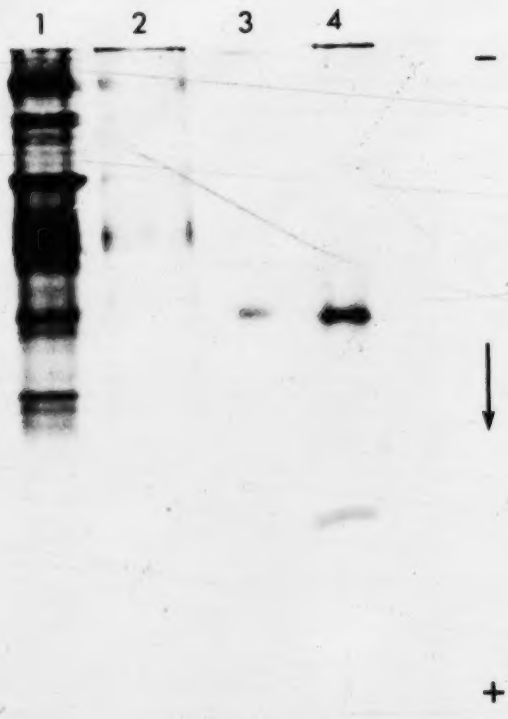


Figure 5. The effect of pre-purifying the load material on the purity of DDC recovered from the immunoaffinity column

Electrophoresis under non-denaturing conditions was carried out on a 10% polyacrylamide slab gel run at 12 ma for 5 hr at 4°C. Slot 1 contained 20 µg of a larval extract partially purified through the alumina step of the conventional procedure. Slot 2 was loaded with 10 µg of protein recovered from fractions 28 to 35 of the elution profile shown in Figure 6a, while slot 3 contained 2 µg of the protein in fractions 67 to 74 of this same profile. Slot 4 shows 8,5 µg of the protein eluted in fractions 67 to 74 from a column loaded directly with crude larval homogenate. The arrow indicates the direction of migration of the proteins.

Successive column runs were performed in which 80, 40, 20 and 30ml, respectively, of alumina load material was passed over the column. When 30ml of alumina eluate was loaded, barely detectable amounts of enzyme activity were evident at fraction 30 while a large peak was present at fraction 68. This was taken as evidence that the column was operating at maximum capacity.

Under the circumstances described above, the purification of DDC to homogeneity was attained for at least 30 runs over a period of several months with typical enzyme activity recovery rates of 40 to 50%(see Table 4 below). Recovery rates were found to gradually decline with the age of the column.

Purification of DDC from Wild Type and Mutant Strains

The fact that all putative DDC structural gene mutants to be analyzed in this investigation produce CRM-positive enzyme (Wright et al., 1976b; Clark et al., 1978) meant that DDC from both wild type and mutant strains could be purified by immunoaffinity chromatography. Details of the purification of each strain are presented in Table 3. A few of the results in the table merit comment.

Firstly, the enzyme activity levels in the crude extracts of the various strains reported in Table 3 do not all closely correspond with those reported in Clark et al.(1978). The likely explanation underlying these deviations concerns the difficulty in accurately staging the large number of organisms collected for these preparations.

Table 3. Purification of dopa decarboxylase from mature larvae of wild type and mutant strains of *Drosophila melanogaster*

STRAIN	PURIFICATION STEP	VOLUME (ml)	PROTEIN CONCENTRATION (mg/ml)	ENZYME ACTIVITY (units/ml)	SPECIFIC ACTIVITY (units/mg)	PURIFICATION (fold)	YIELD (%)
C.S.	Crude extract	1,010	5.4	14.8	2.7	1.00	100
	Alumina gel eluate	87	0.91	37.9	42	15.6	22
	IA eluate	0.22	0.61	3010	4900	1810	4.4
$\frac{Ddc^5}{CyO}$	Crude extract	970	6.9	7.49	1.1	1.00	100
	Alumina gel eluate	87	1.1	19.4	18	16.4	23
	IA eluate	0.22	0.41	1240	3000	2730	3.8
$\frac{Ddc^8}{CyO}$	Crude extract	970	6.6	6.54	0.99	1.00	100
	Alumina gel eluate	87	1.1	15.2	14	14.1	21
	IA eluate	0.26	0.20	446	2200	2220	1.8
$\frac{Ddc^5}{Ddc^8}$	Crude extract	790	7.5	1.21	0.16	1.00	100
	Alumina gel eluate	70	1.1	1.42	1.3	8.13	10
	IA eluate	0.27	0.31	130	420	2630	3.7

Therefore, the C.S. organisms used in this purification could well have been closer to mid-third instar than late third instar, accounting for their apparently lower than expected DDC activity relative to Ddc^{5}/CyO and Ddc^{8}/CyO.

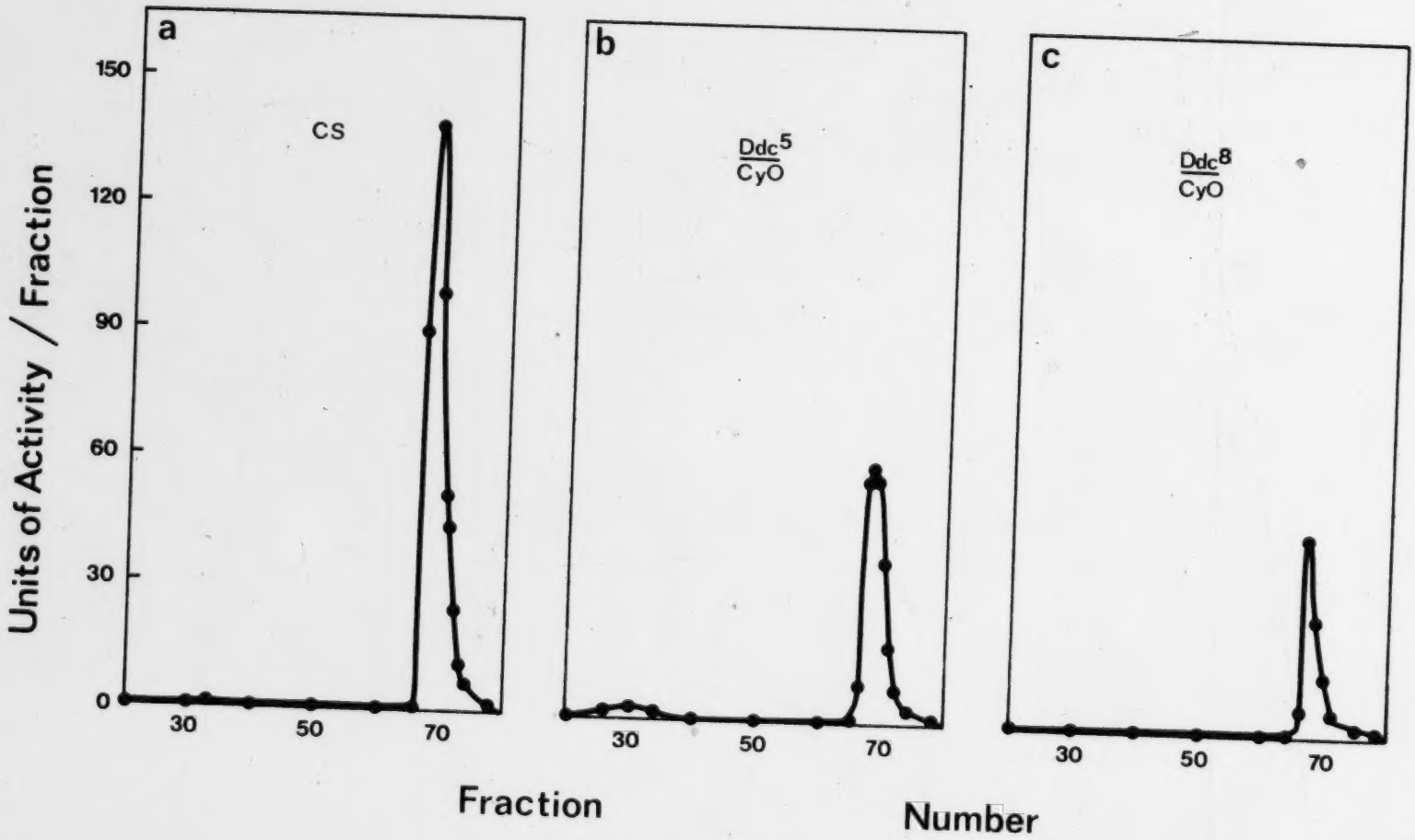
Secondly, the specific activity of the C.S. enzyme purified by the IA procedure is approximately 20% higher than wild type enzyme purified by the conventional procedure reported by Clark et al.(1978). This is a reasonable expectation considering the fact that the conventional procedure involves three separate columns following the alumina preparation, each requiring a day to run, whereas the IA procedure involves only one column requiring about 12hr. Subjecting an enzyme to more lengthy and more numerous column procedures, as is necessary in the conventional procedure, might well be expected to reduce both the specific activity and yield of the purified enzyme relative to the one step procedure involving the IA column.

Thirdly, the yield for all strains was comparable except for Ddc^{8}/CyO which showed about one half the yield of the other strains. Since the elution profile for Ddc^{8}/CyO was not dramatically different from the Ddc^{5}/CyO profile, one may conclude that the loss of Ddc^{8}/CyO occurred in the subsequent and final step of ultrafiltration. It should be noted that ultrafiltration consistently resulted in at least a 50% loss of yield in all cases.

Figure 6 shows the elution profiles of C.S., Ddc^{5}/CyO and Ddc^{8}/CyO. The Ddc^{5}/Ddc^{8} material was treated in a

Figure 6. Immunoaffinity chromatography of DDC from extracts of wild type and mutant strains

A column was prepared by layering 6.2 ml(1.2 x 5.5 cm) of antibody-Sepharose on top of 77 ml(1.2 x 68 cm) of Sephadex G-25. The protocol for eluting purified DDC was as follows: At least 300 ml of equilibration buffer (0.01 M sodium phosphate, pH 7.3, containing 1.3 mM 2-mercaptoethanol) was initially passed through the column followed by the application of 27.5 ml of larval extract which had been pre-purified through the alumina step. Subsequently, 50 ml of 1 M NaCl in 0.01 M sodium phosphate, pH 7.3, containing 4mM 2-mercaptoethanol followed by 120 ml of 10% glycerol in 0.01 M sodium phosphate, pH 7.3, containing 4 mM 2-mercaptoethanol were applied. DDC was eluted with 40 ml of 3 M NH_4SCN in 0.01 M sodium phosphate, pH 7.3, containing 4 mM 2-mercaptoethanol and 10% glycerol. Fractions of 60 drops each with an average volume of 3.9 ml were collected from the time that the load material was initially applied. The flow rate was maintained at 30 ml/hr. All operations were performed at 4°C.


a
b
c
CS
Ddc5/CyO
Ddc8/CyO
Units of Activity / Fraction
150
120
90
60
30
0
30
50
70
Fraction Number

somewhat different manner and therefore is not directly comparable. A partial purification of 200g of larvae through the alumina step typically resulted in 80 to 90ml of eluate. Instead of applying approximately one third of the alumina preparation to the IA column, as was done for the other strains, the entire Ddc^5/Ddc^8 alumina preparation was loaded at once. This was done since Clark et al. (1978) had shown that Ddc^5/Ddc^8 had only 15% of the wild type level of CRM. Unexpectedly, the column behaved as if overloaded. The DDC in excess of column capacity in the first run was reapplied for a second run. This procedure was continued until, by the fourth run, no further enzyme activity was evident in the fractions where pure DDC is usually found. These results suggested that the amount of CRM determined by the precipitin reaction used by Clark et al. (1978) does not necessarily reflect the CRM bound by the antibody-Sepharose of the IA column. Lowry protein determinations and analytical gels, about to be described, later substantiated the finding that far more CRM was recovered in this purification of Ddc^5/Ddc^8 than had initially been anticipated.

The recovery of DDC activity from the IA column for the various strains (Table 4) was remarkably similar except in the case of Ddc^5/Ddc^8. However, this exception is expected if indeed the column was initially overloaded, as was suggested above. All runs shown in the table were performed on the same new preparation of antibody-Sepharose.

Table 4. Recovery of DDC activity from immunoaffinity chromatography

STRAIN	COLUMN RUN	PROTEIN APPLIED TO IA COLUMN (mg)		RECOVERY OF DDC ACTIVITY (% of activity loaded)
C.S.	1	25.0		46.7
	2	12.7		56.6
	3	26.4		43.8
			Average	49.0
$\frac{Ddc^5}{CyO}$	1	29.4		50.3
	2	30.5		39.6
	3	29.4		46.3
			Average	45.4
$\frac{Ddc^8}{CyO}$	1	29.5		47.3
	2	30.0		36.9
	3	29.5		39.0
			Average	41.1
$\frac{Ddc^5}{Ddc^8}$	1	72.3		19.7

All of the above purification runs were performed using DDC which had been partially purified through the alumina step of the conventional purification procedure. This material was subsequently applied to the immunoaffinity column. The same preparation of antibody-Sepharose was used in all runs presented here.

C. Analysis of Putative DDC Structural Gene Mutants

Peptide Mapping

The potential for purifying mutant enzyme by the IA column led to attempts to detect physical differences among the gene products of the putative DDC structural gene mutant strains being studied. One approach to this problem was two-dimensional peptide mapping described by Bates *et al.* (1975).

Briefly, the protocol, which is described in the Materials and Methods, included unfolding the native structure of the protein to facilitate the digestion into tryptic and chymotryptic peptides. The digest was then electrophoresed in one dimension and chromatographed in a second dimension. Although the peptide map obtained by the authors for rabbit muscle aldolase was reproduced satisfactorily (Figure 7), attempts to produce a well resolved map for wild type DDC were unsuccessful.

The major obstacle encountered was that the IA column was capable of producing only 100ug of pure DDC per run. Since a peptide map required 50ug, the purification of sufficient wild type enzyme for refining the technique as well as the purification of mutant enzyme for analysis was considered impractical. The method was therefore set aside until the time that it might be repeated using radiolabelled enzyme.

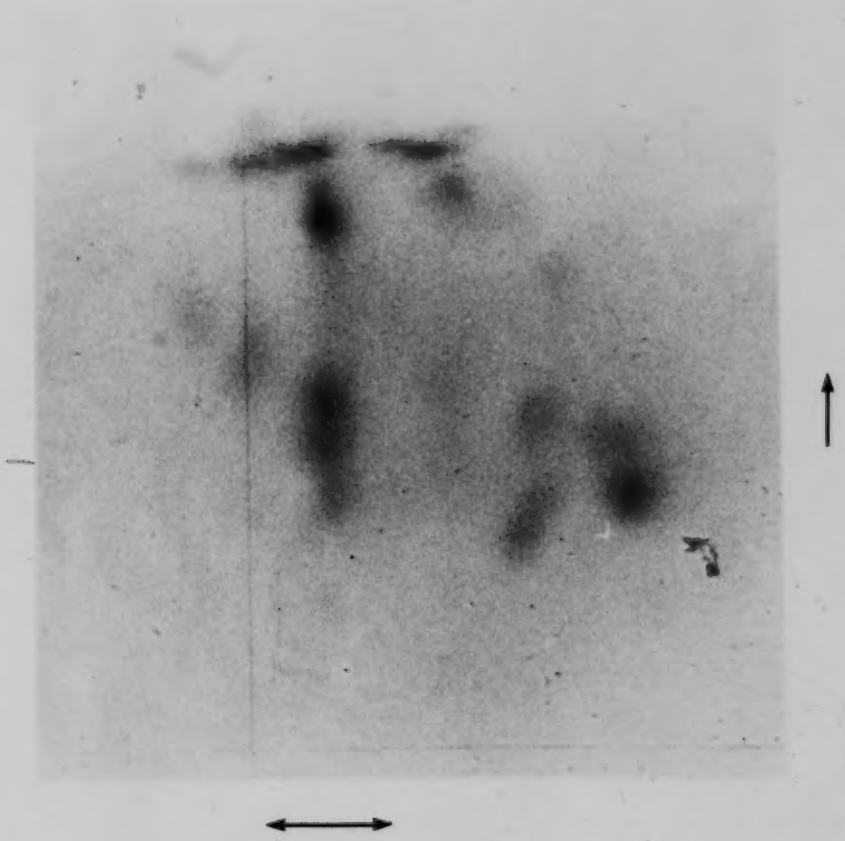


Figure 7. Two-dimensional peptide map of rabbit muscle aldolase

A tryptic and chymotryptic digest of rabbit muscle aldolase was prepared as described in the Materials and Methods. A 50 µg sample was applied to the cellulose thin layer chromatography support shown in the photograph and subsequently electrophoresed at 500 V for 20 min at 4°C as indicated by the horizontal arrows. Following drying, the digest was chromatographed in the direction of the vertical arrow. Visualization of the resulting peptides was accomplished by a ninhydrin-cadmium stain.

Electrophoretic Analysis of DDC from Wild Type and Mutant Strains

The IA-purified DDC from C.S., Ddc^5/CyO, Ddc^8/CyO and Ddc^5/Ddc^8 was analyzed on three kinds of polyacrylamide slab gels in an attempt to detect electrophoretic variation among DDC samples from the putative structural gene mutants. The gel run under non-denaturing conditions compared the relative mobility of the native enzyme from the four strains, while the SDS gel demonstrated the migration of enzyme subunits on the basis of molecular weight only. Finally, the urea gel separated variants according to both the molecular weight and net charge of structurally unfolded enzyme subunits.

Figure 8 presents the result of electrophoresis of native DDC from the wild type and three mutant strains under non-denaturing conditions. With the exception of Ddc^5/Ddc^8 in slot 2, which indicated a poorly resolved slow species relative to the sharp major band, all strains showed a pronounced single band with a common relative mobility. Thus, this electrophoretic technique did not detect any differences which might exist between the two types of enzyme postulated to be present in these mutant heterozygotes(Figure 1).

The SDS gel in Figure 9 indicated that the polypeptides produced by both mutant and wild type genes have similar molecular weights. Several additional protein species of molecular weights less than DDC were evident that did not

Figure 8. Electrophoretic analysis of purified DDC from wild type and mutant strains on non-denaturing gels

A 10% polyacrylamide slab gel was prepared and run according to the protocol outlined in the Materials and Methods. Slots 1 to 5 contained IA-purified DDC from C.S., Ddc^5/Ddc^8, Ddc^8/CyO, Ddc^5/CyO, and C.S., respectively. Approximately 3 µg of protein was loaded in each slot. The arrow indicates the direction of migration of the proteins.

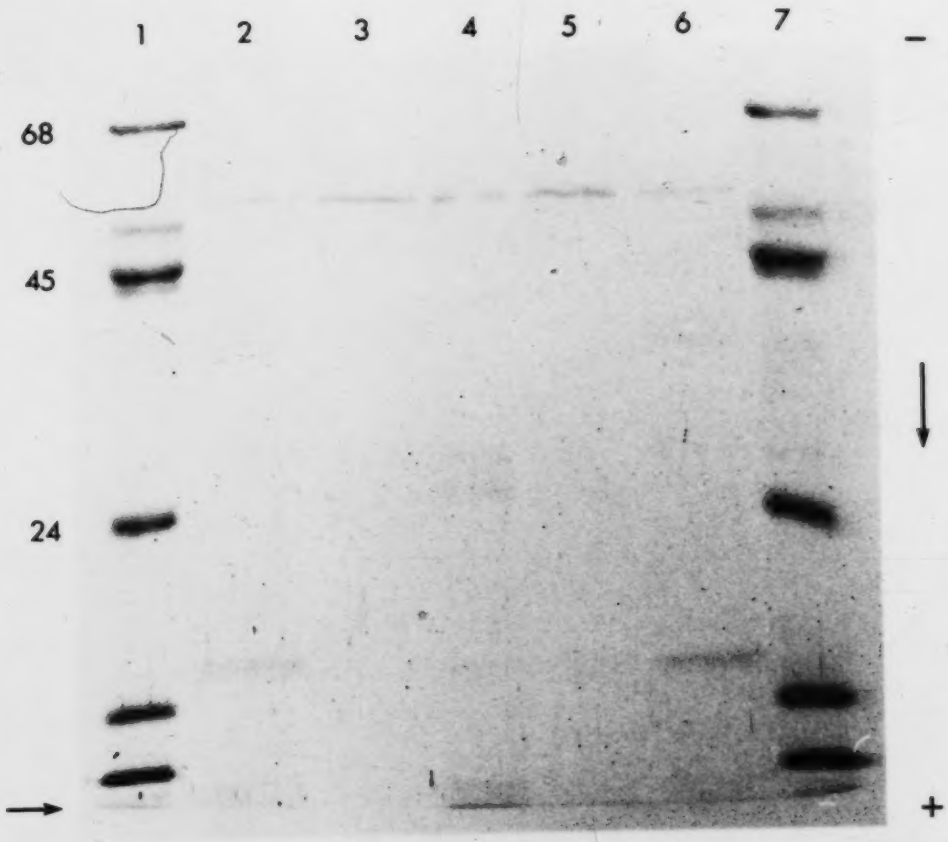


Figure 9. Electrophoretic analysis of purified DDC from wild type and mutant strains in the presence of SDS

A 10% SDS slab gel was prepared as described in the Materials and Methods and run at 90 V for 3.5 hr at room temperature. Slots 1 and 7 contained 15 µg of Dalton Mark VI molecular weight standards (Sigma Chemical Co.) and the corresponding molecular weights (x 10^{-3}) are indicated in the left margin. Slots 2 to 6 contained IA-purified DDC from C.S., Ddc^{5}/CyO, Ddc^{8}/CyO, Ddc^{5}/Ddc^{8} and C.S., respectively. These samples were from the same preparations run on the gel in Figure 8. Approximately 3 µg of protein was loaded in each slot. The vertical arrow indicates the direction of migration of the proteins while the horizontal arrow marks the position of bromophenol blue which was used as a tracking dye.

appear in the non-denaturing gel in Figure 8. The presence of these smaller molecular weight fragments might be attributed to degradation of the enzyme. Unfortunately, all of the samples were stored for several weeks at 4°C prior to these gel runs. Apparently the strand scissions which are postulated to have occurred only became evident upon denaturation in SDS.

A urea gel was developed to reveal electrophoretic differences that might exist between the denatured mutant and wild type polypeptides. Since the isoelectric point of native DDC has been reported to be 4.9(Clark et al.,1978), urea gels at pH 8.0 were run initially using the protocol of Perrie and Perry(1970). While ovalbumin and yeast ADH standards migrated into the gel, DDC appeared to precipitate on the well surface. That is, at pH 8.0 and electrophoresis towards the anode, the enzyme did not migrate in either direction. Such a result suggested that the unfolded DDC subunit had an isoelectric point close to pH 8.0 and therefore a buffer system of lower pH was used. Attempts to adapt a procedure described by Creighton(1979) using a Tris-acetate buffer system at pH 4.0 resulted in poor band resolution. Ultimately, the method outlined by Panyim and Chalkley(1969) produced the most acceptable resolution of the enzyme in the gel. Migration occurred towards the cathode, but at a rate which suggested the total net charge on the subunits was small.

The urea gel resulting from electrophoresing wild type

and mutant enzyme with the sample loading conditions as described by Panyim and Chalkley(1969) was complicated(Figure 10a). The results have been interpreted according to the hypothesis of Clark et al. (1978)(also see Figure 1). Slots 1 and 5 indicated that in the wild type one pronounced band was resolved(F). This is assumed to be the wild type subunit. In slot 2, in which DDC from Ddc^5/CyO was run, band F was apparent, but the most pronounced band was a slower migrating species(S) which is assumed to be the Ddc^5 subunit. Neither S nor F was resolved in Ddc^8/CyO(slot 3). In the Ddc^5/Ddc^8 preparation(slot 4), band S was present but F was missing. It seemed as though the Ddc^8 species had migrated to the same position as Ddc^5 or, alternatively, had precipitated on the well surface.

In an attempt to distinguish between these alternatives Ddc^5/CyO and Ddc^5/Ddc^8, were run on a second gel. With the notion that the precipitate on the well surface was due to the low pH in the sample (pH less than 3), the sample preparation of Panyim and Chalkley(1969) was abandoned in favour of one with a higher pH(4.1) which included dissolution of solid urea to a final concentration of 8M into the enzyme preparation with the subsequent addition of acetic acid to a final concentration of 5%(v/v). This gel is shown in Figure 10b. The precipitate at the well surface was much reduced. Further, the additional bands common to all slots in Figure 10a were absent suggesting these were also caused by the exposure of the sample to a low pH during

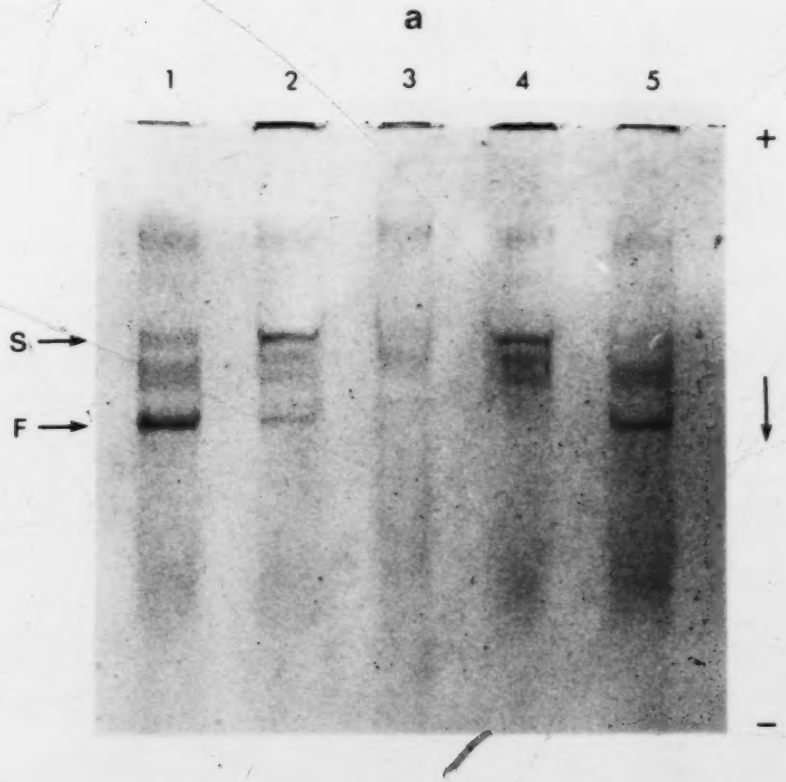
Figure 10. Electrophoretic analysis of purified DDC from wild type and mutant strains in the presence of urea

All gels shown in these photographs were 8 M urea polyacrylamide slabs prepared as described in the Materials and Methods. Approximately 9 µg of purified DDC was loaded in each slot. The vertical arrow indicates the direction of migration of the proteins. Two prominent bands, which are common throughout the photographs, are indicated as slow(S) and fast(F) species. These are discussed at length in the text.

a. The sample loading procedure of Panyim and Chalkley(1969) was used in this gel(15% sucrose and 5% acetic acid) which resulted in a pH of less than 3. Slots 1 to 5 contained purified DDC from C.S., Ddc^5/CyO, Ddc^8/CyO, Ddc^5/Ddc^8 and C.S., respectively. The interpretation of this gel suggested that this loading procedure was excessively harsh.

b. The sample load was modified in this gel from that in (a) to include 8 M urea and 5% acetic acid bringing the pH of the loading buffer to 4.1. Slots 1 and 2 were loaded with DDC from Ddc^5/CyO and Ddc^5/Ddc^8, respectively. Material from C.S. was not available for this run.

c. The sample loading conditions in this gel were the same as in (b). However, in this run, slot 1 contained DDC from C.S. and slot 2 was loaded with DDC from Ddc^5/Ddc^8. Only band F is evident in C.S. while Ddc^5/Ddc^8 shows indications of both S and F species(as in gel b), although these are not well resolved.


a
1
2
3
4
5
+
S
F
−
b
1
2
+
S
F
−
c
1
2
+
S
F
−

loading. The Ddc^5/CyO sample(slot1) showed the same banding pattern as in slot 2 of Figure 10a while Ddc^5/Ddc^8 appeared to be identical to Ddc^5/CyO. In a third gel(Figure 10c) the wild type enzyme was loaded under the higher pH conditions and was resolved, as before, into the band designated F. On the same gel, enzyme from Ddc^5/Ddc^8 was again run and, although poorly resolved, indicated two bands just as it had shown in Figure 10b.

Repeated attempts were made to resolve the subunits from the Ddc^8/CyO strain on urea gels without success. The primary difficulty was the inability to obtain sufficiently concentrated material.

In Vivo Labelling of Proteins in Larvae

The difficulties in performing analyses on the small amounts of pure DDC obtained by either of the procedures used in this thesis have been mentioned. For this reason, an attempt was made to obtain radiolabelled DDC.

As outlined in the Materials and Methods, larval proteins were radioactively labelled by feeding a prescribed volume of an aqueous solution containing radioactive amino acids to third instar larvae. Under these conditions, Figure 11a indicates that the maximum incorporation of radioactivity had occurred by about 10hr, at which time a crude extract was prepared. Also shown in this graph is that the amount of radioactivity incorporated into larval proteins is directly proportional to the amount of radioactivity available to the larvae in the amino acid

Figure 11. *In vivo* labelling of proteins in third instar larvae

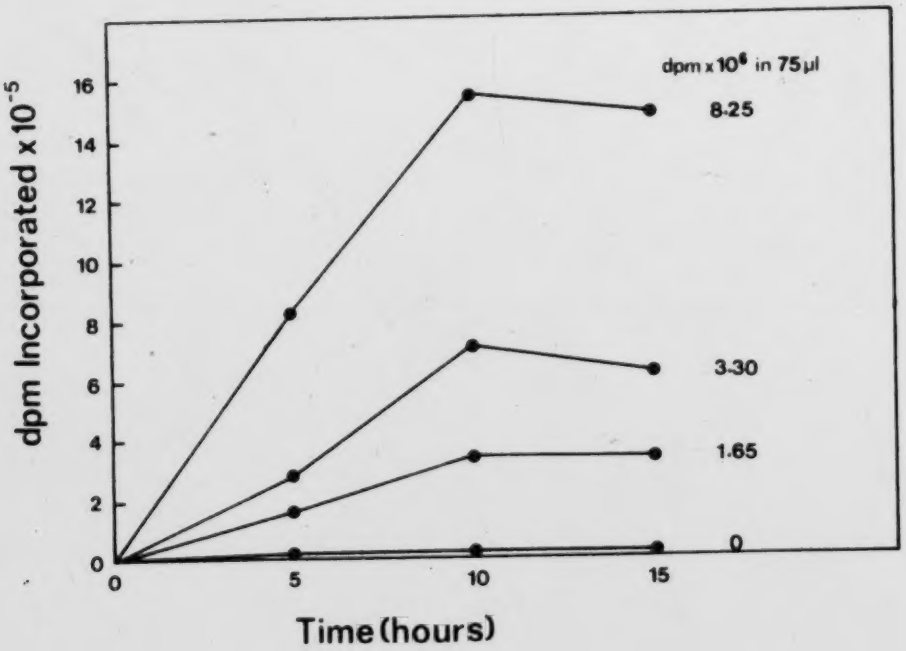
a. Time course of incorporation of radioactive label into proteins

Third instar larvae(35 to 40 larvae/50 mg) were fed variable amounts of a ^{14}C-labelled amino acid mixture, details of which are given in the Materials and Methods. At 5, 10 and 15 hr following the initiation of feeding, a crude extract was prepared and the amount of ^{14}C that had been incorporated was determined by aliquots which had been TCA-precipitated.

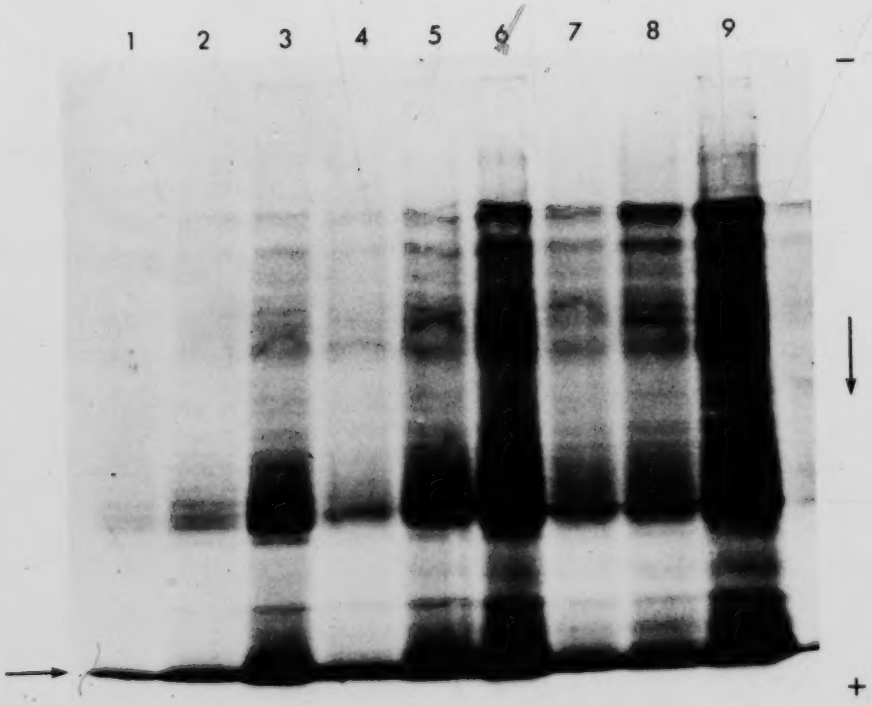
b. Electrophoretic analysis of the labelled proteins

A 10% SDS gel was prepared as described in the Materials and Methods and run at 90 V for 3.5 hr at room temperature. Following destaining, the gel was fluorographed(see Materials and Methods) with an 8 day exposure. Samples of 10 µl from extracts of larvae fed a total of 1.65×10^6 dpm for 5, 10 and 15 hr were run in slots 1, 4 and 7, respectively. Similarly, extracts from larvae fed a total of 3.30×10^6 dpm for 5, 10 and 15 hr were run in slots 2, 5 and 8, respectively. Finally, extracts from larvae fed a total of 8.25×10^6 dpm for 5, 10 and 15 hr were run in slots 3, 6 and 9, respectively. These samples correspond to the time points in the graph above.

a


dpm x 10⁶ in 75 µl
8·25
3·30
1·65
0
dpm Incorporated x 10⁻⁵
Time (hours)
0
2
4
6
8
10
12
14
16
0
5
10
15

b


1
2
3
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5
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7
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9
−
+

mixture. The fluorograph in Figure 11b indicates that a wide variety of proteins, including several of molecular weights comparable to DDC, had incorporated radioactively labelled amino acids. Conclusive evidence demonstrating that DDC was among those labelled proteins synthesized during this procedure has now been obtained(Kraminsky, 1979).

IV. DISCUSSION

A. Genetic Implications of the Homodimeric Structure of DDC

The electrophoresis of DDC under non-denaturing conditions yielded a molecular weight of 102,200 while the molecular weight by sucrose gradient sedimentation was 112,600. These results, in conjunction with the finding that when DDC is electrophoresed in the presence of SDS a single band is evident corresponding to a molecular weight of 54,000, suggest that this enzyme in Drosophila is a homodimer. Additionally, DDC from the wild type demonstrated a single prominent band when electrophoresed in the presence of 8M urea. Thus, even when the migration of the subunits of the enzyme is dependent upon both size and charge, only one subunit species is evident.

The finding that DDC from Drosophila is a homodimer has immediate implications concerning the genetic system for this enzyme. Wright et al. (1976b) hypothesized that the 1(2)Ddc$^+$ gene might code for a catalytic subunit of the enzyme, while the 1(2)amd$^+$ gene might code for a subunit having an allosteric site involved in the regulation of the enzyme. The results reported in this thesis make this hypothesis unlikely. In fact, the biochemical and genetic data available at this time fail to indicate any direct functional relationship between the 1(2)Ddc and 1(2)amd genes. Clark et al. (1978) proposed that these genes might be the result of an ancient duplication event with the function

of their gene products diverging with time. They suggested that a possible function for the l(2)amd gene product is the decarboxylation of 5-hydroxy-tryptophan.

The result indicating a homodimeric structure for DDC from Drosophila contrasts to the finding of Fragoulis and Sekeris(1975c) whose results on SDS gels indicated that the enzyme from Calliphora is a heterodimer. In view of the fact that their purification procedure was virtually identical to the conventional procedure used here, one must question their results. It was only after a careful pooling of the fractions from the final chromatography step of the conventional purification that we managed to eliminate the contaminating species which had given rise to our erroneous observation(cited by Wright et al.,1976b) that two subunits had been resolved on SDS gels. Further doubt about their earlier observation is raised by a later report(Fragoulis and Sekeris, 1975b) that only a single species was evident as a result of electrophoresis in the presence of SDS following the cell-free translation of RNA from late third instar larvae which had been immunoprecipitated with antibody raised against DDC.

B. Analysis of Putative DDC Structural Gene Mutants

The analysis of the putative DDC structural gene mutants isolated by Wright et al.(1976b) was undertaken using the complementing alleles, Ddc^{5} and Ddc^{8}. Since the cross of Ddc^{5}/CyO x Ddc^{8}/CyO did yield viable flies

possessing heterczygosity for the mutant alleles at the Ddc locus, it was anticipated that these structural gene mutants did not involve gross changes in the structure of the enzyme. Instead, a more likely situation was that one or more amino acid substitutions had occurred, some of which might produce electrophoretic variation relative to the wild type. Consequently, electrophoretic variation among these strains(C.S., Ddc^5/CyO, Ddc^8/CyO, and Ddc^5/Ddc^8) was not expected following migration under denaturing conditions in the presence of SDS and none was found(Figure 9). Unfortunately, electrophoresis of the native enzyme also failed to demonstrate electrophoretic variation(Figure 8). It was then felt that electrophoresis in the presence of 8M urea might distinguish between mutant and wild type subunits since this technique denatures the enzyme and then unfolds the subunits, exposing internal charge differences that might have been masked under the conditions of Figure 9.

The electrophoresis of DDC from the wild type and mutant strains in the presence of 8M urea was interpreted in the Results according to the hypothesis of Clark et al.(1978). This interpretation suggested that the Ddc^5 allele produces a subunit which is an electrophoretic variant relative to the wild type, but that the subunit produced by the Ddc^8 allele is not a variant. It is evident from Figure 10(a and c) that the wild type subunit migrated slowly under the running conditions indicating that its net charge is probably small. Consequently, it is not surprising

that a single charge alteration might cause the substantial difference in migration between the two types of subunits that was observed.

Due to the technical difficulties in obtaining sufficient material for analysis, a number of techniques have been developed which should provide better data upon which to decide whether or not *Ddc*^{5} produces an altered subunit which is detectable by electrophoresis.

The ability to obtain radiolabelled DDC by the *in vivo* method should circumvent the problem of insufficient quantities of enzyme for electrophoretic analysis on 8M urea gels. The labelled enzyme can be purified from crude extracts in one of two ways. Two successive passages of the labelled crude extract over the IA column, as will be described in more detail in the text to follow, should generate purified, labelled DDC. Alternatively, labelled DDC which is complexed with antibody against DDC may be precipitated by *Staphylococcus aureus* cells which are known to have an affinity for IgG molecules(see Goding,1978 for review). The DDC is dissociated from its antibody by treating with SDS or 8M urea. Hodgetts(personal communication) has demonstrated that the latter technique results in a single band from each of the putative DDC structural gene mutants migrating to a common position following electrophoresis in the presence of SDS. The results using this technique to analyze the strains of interest in the presence of 8M urea are pending.

Thus, the *in vivo* labelling of larval proteins and the development of an electrophoretic method for analyzing the enzyme in the presence of 8M urea are techniques which should facilitate the screening of a number of *l(2)Ddc* mutants for evidence of electrophoretic variation in a mutant subunit. In addition to providing convincing evidence that the DDC-deficient mutants isolated by Wright *et al*,(1976b) are structural gene mutants, the demonstration that one of these stocks possesses an electrophoretic variant would be of benefit in establishing that the DDC overproducers and underproducers(Estelle and Hodgetts, 1979) are the result of mutations in a cis-acting regulatory site adjacent to the structural gene, analogous to the situation described for the *rosy* locus(Chovnick *et al*.,1976).

C. The Potential of the Immunoaffinity Column

The IA column used in this study was effective in purifying CRM-positive enzyme from mutant and wild type strains. However, difficulties were continuously encountered in the analysis due to the limited quantities of enzyme which could be purified. It is obvious that future studies of this enzyme will be greatly facilitated by a substantial increase in the scale of the IA column used here.

The major restrictions in the capacity of an IA column to be prepared for enzyme purification rest with the quality and quantity of antibody available. With the large amount of antibody now available, it is possible to prepare an IA

column with a large capacity for producing highly purified DDC. Further, the purification of a sufficient quantity of DDC by the column would enable the preparation of a DDC-Sepharose column to purify, from crude IgG, antibody directed against DDC. The availability of sufficient quantities of highly purified antibody would then permit an antibody-Sepharose column to be prepared capable of even larger scale purifications.

Although the immunoaffinity chromatography technique was performed in this study on pre-purified material for reasons dscribed in the Results, it is practical to purify DDC on a small scale by successive passages of a crude extract over two IA columns. This method is currently being used in an attempt to purify labelled enzyme from the crude mixture of *in vivo* labelled proteins which will permit a radioimmune assay to be developed to detect CRM(M. Estelle, personal communication).

Finally, the IA column prepared from antibody raised against *Drosophila* DDC might provide a useful tool to compare the similarity between DDC from *Drosophila* and from *Calliphora*. If the IA column retained the *Calliphora* enzyme, the subunit structure of this enzyme could be analyzed electrophoretically on SDS gels to resolve the ambiguity which exists concerning its structure.

The study undertaken in this thesis stresses the requirement for constant interplay between genetical analysis and biochemical analysis that is essential in the

investigation of questions in molecular biology. Hopefully, the results presented herein will contribute to additional insight into the regulation of gene expression in higher eukaryotes.

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APPENDIX

Preparation of Polyacrylamide Gels

1. NON-DENATURING SLAB GELS

Stock Solutions:

Stock A

1 N HCl 48 ml
titrate with Tris base to pH 7.9
TEMED 0.46 ml
water to 100 ml

Stock B

0.48 M imidazole-HCl, pH 5.7

Stock C

acrylamide 30 g
BIS 1 g
water to 100 ml

Stock D

acrylamide 10.0 g
BIS 2.5 g
water to 100 ml

Stock E

riboflavin 4 mg
water to 100 ml

Stock F

sucrose 40 g
water to 100 ml

Preparation of Bottom(Separating) Gel:

%gel	water(ml)	Stock C (ml)	Stock A (ml)
8.5	2.2	6.8	3.0
10.0	1.0	8.0	3.0
10.5	0.6	8.4	3.0

Dilute this 12 ml of solution with an equal volume of 0.14% ammonium persulfate freshly prepared(28 mg/20 ml water).
Pour the gel immediately.
Polymerization should occur within 30 to 45 min.

1. NON-DENATURING SLAB GELS continued

Preparation of Top(Stacking) Gel(2.5%):

1 ml Stock B
2 ml Stock D
1 ml Stock E
2 ml Stock F
2 ml water
4.5 µl TEMED

Pour and polymerize for 45 to 60 min at a distance of 6 in from a light box.
The gel is not pre-run.
Samples are loaded in 10% glycerol.
The reservoir buffer is 0.034 M asparagine neutralized to pH 7.3 with Tris base.
The gel is run at 12 ma for 5 hr at 4°C toward the anode with reservoir buffer changes every 1.5 hr.

2. DENATURING SDS SLAB GELS

Stock Solutions:

Lower gel stock
1.5 M Tris-HCl, 0.4% SDS, pH 8.8

Upper gel stock
0.5 M Tris-HCl, 0.4% SDS, pH 6.8

30% acrylamide stock

acrylamide	30 g
BIS	1 g

2. DENATURING SDS SLAB GELS continued

Preparation of the Gel:

Stock	10% Lower Gel (ml)	4.5% Upper Gel (ml)
Lower gel	6.0	---
Upper gel	---	1.9
30% acrylamide	8.0	1.1
water	10.0	4.5
10% ammonium persulfate	0.12	0.023
TEMED	0.011	0.008

Gels polymerize within 45 min at room temperature.
Samples are diluted 1:1 with reducing buffer(30 ml of 10% SDS in water; 12.5 ml of upper gel stock; 5 ml of 2-mercaptoethanol; and 10 ml of glycerol) and are boiled for 5 min prior to loading.
One litre of reservoir buffer consists of 3 g of Tris base, 14.4 g of glycine, and 1 g of SDS which are dissolved in water.
The final pH of the reservoir buffer should be 8.1.

3. DENATURING 8 M UREA GELS

Mix the following ingredients in the order listed:

10 M urea	24.0 ml
water	0.8 ml
glacial acetic acid	1.5 ml
acrylamide	3.0 g
BIS	20.0 mg
TEMED	100.0 µl

The solution is polymerized with the addition of 1 ml(16 mg/ml) of ammonium persulfate.
Allow 2 hr for polymerization.
The gel is pre-run at 150 V until the current remains constant, usually 2 to 4 hr.
Samples are loaded in 8 M urea containing 5% acetic acid.
The gel is run at 150 V for 12 hr at 4°C towards the cathode.

Ninhydrin-Cadmium Stain

Stock Solutions:

Stock A

1% ninhydrin in acetone(20g/2l)

Stock B

water	500 ml
acetic acid	250 ml
cadmium acetate	5 g

The stain is prepared just prior to use by mixing 100 ml of Stock A with 15 ml of Stock B.